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EXPERT REVIEW

RNA Interference and Cancer Therapy

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ABSTRACT Since its discovery in 1998, RNA interference (RNAi) has revolutionized basic and clinical research. Small RNAs, including small interfering RNA (siRNA), short hairpin RNA (shRNA) and microRNA (miRNA), mediate RNAi effects through either cleavage-dependent or cleavage-independent RNA inducible silencing complex (RISC) effector processes. As a result of its efficacy and potential, RNAi has been elevated to the status of "blockbuster therapeutic" alongside recombinant protein and monoclonal antibody. RNAi has already contributed to our understanding of neoplasia and has great promise for anti-cancer therapeutics, particularly so for personalized cancer therapy. Despite this potential, several hurdles have to be overcome for successful development of RNAi-based pharmaceuticals. This review will discuss the potential for, challenges to, and the current status of RNAi-based cancer therapeutics.

KEY WORDS cancer therapy · delivery · RNA interference

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INTRODUCTION

Cancer is a disease characterized by aberrant cell repair, differentiation, proliferation, and cell to cell/tissue/host interaction. It is a result of long-term accumulation of genetic and epigenetic alterations. Despite apparent lineage based phenotypic homogeneity there is both significant intra- and inter-tumoral heterogeneity as well as intra- and inter-patient heterogeneity arising from the evolving genetic, epigenetic and regional adaptive patterns. This molecular heterogeneity underlies the distinctly different responses to standard systemic therapeutic approaches. As a result, there is increasing pressure to characterize molecular profiles of individual patients and to use this information to develop personalized therapy programs. RNA interference (RNAi), due to its specificity, adaptability and breadth of targeting capability, has great potential to serve as a personalized gene therapy for cancer.

RNAi, discovered by Fire and Mello in 1998 (1), is defined as a mechanism of gene-silencing produced by small RNAs, which include endogenous microRNA (miRNA) and exogenous siRNA or shRNA. This gene-silencing is an evolutionarily conserved process and is highly dependent on gene sequence. Due to the inherent difficulties of inhibiting potential targets with small molecular drugs, recombinant proteins and monoclonal antibodies, researchers and clinicians have looked towards RNAi as a revolutionary approach to target "undruggable" targets with robustness and specificity.

The mechanism of RNAi has been thoroughly investigated. Briefly, a double-stranded small RNA is incorporated into the pre-RISC (RNA Induced Silencing Complex) followed by the cleavage-dependent (in the case of matched guide and passenger strands) or—independent (unmatched guide and passenger strands) release of the passenger strand

forming the guide strand containing RISC. The guide-strand (anti-sense strand) guides RISC to the complementary or near-complementary region of target mRNA. In general, siRNA (small interfering RNA from the cleavage-dependent RISC) with a perfect match to its target cleaves the target mRNA via the endonuclease Ago2 whereas miRNA (micro-RNA), with an imperfect match to its target, induces mRNA degradation (or sequestration in the p-body (processing body) and translational inhibition. Duplex siRNA and vector-encoded shRNA were first introduced as a way to silence gene expression in animals in 2002 (2,3).

siRNA, shRNA, miRNA AND bi-shRNA

siRNA

Synthetic siRNA was the first RNAi technology to be introduced into mammalian cells in order to accomplish sequence specific gene silencing (4). This type of small RNA directly incorporates into RISC, where its guide-strand binds to and cleaves the complementary mRNA. When the cleaved mRNA is released and further degraded, the guide-strand-bound RISC binds to another mRNA and starts a new cycle of cleavage (Fig. 1). siRNA is able to cleave target RNA in both the cytoplasm and the nucleus (5). As a result of this efficient suppression machinery, a well-designed siRNA is capable of inhibiting gene expression at picomolar concentrations *in vitro*. However, there are limitations to *in vivo* use. First, siRNA is sensitive to nucleases present in plasma and therefore must be protected for delivery to target tissues. Second, frequent dosing is required since there is no endogenous production of the delivered "therapeutic" and siRNA pharmacokinetics are characterized by a short half-life as well as rapid clearance. Therefore, chemical modification is required to improve stability and circulation half-life.

shRNA

As an alternative strategy to siRNA, short hairpin RNA (shRNA) has been developed to allow for long-term gene silencing (6–8). shRNA is transcribed in the nucleus from an expression vector bearing a short double-stranded DNA sequence with a hairpin loop. The shRNA transcript is then processed and loaded into RISC in the cytoplasm, following the same cytoplasmic RNAi process as siRNA. Likewise, the extent of homology between the guide strand and the target

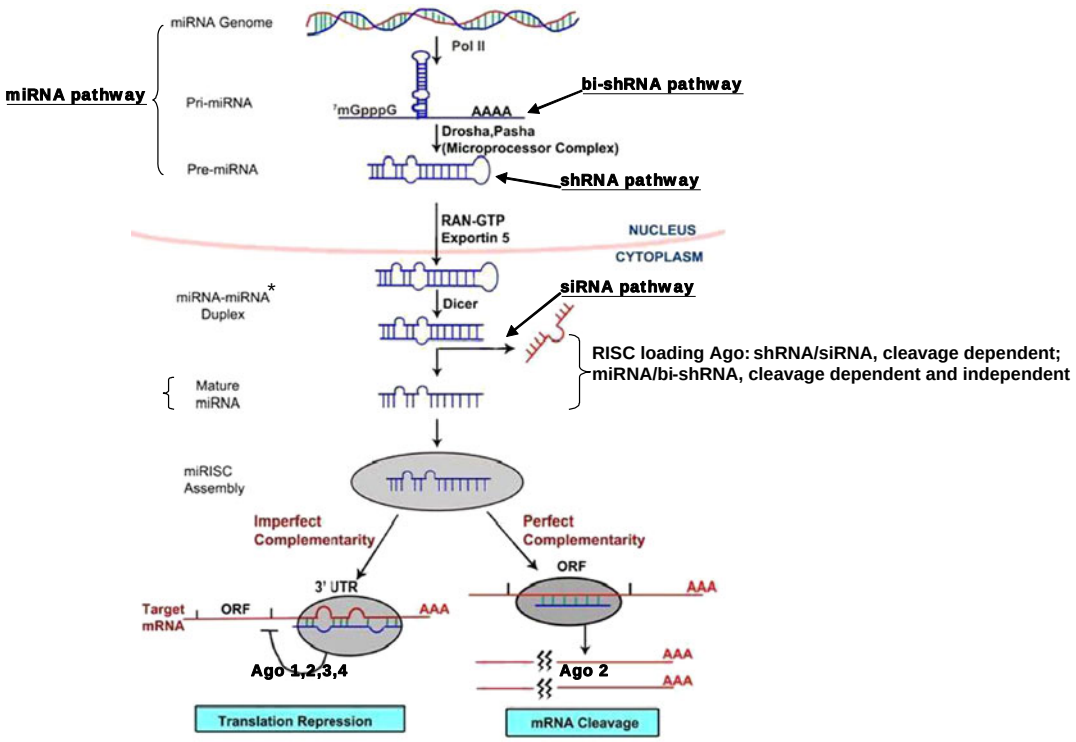

Fig. 1 Schematic presentation of siRNA, shRNA, miRNA and bi-shRNA pathways. After being processed by microprocessor complex in the nucleus, pre-miRNA is exported to the cytoplasm and then cleaved by Dicer to produce a miRNA duplex. Single-stranded matureshRNA is loaded into RISC to either induce mRNA cleavage or translation repression based on the degree of complementarity. Bi-shRNA exploits the miRNA pathway and produces a cleavage-dependent unit and a cleavage-independent unit. After loading into RISC, the two units elicit mRNA cleavage and translation inhibition respectively. shRNA is transcribed in the nucleus, processed by Dicer and loaded into RISC in the cytoplasm. Synthetic siRNA directly incorporates into RISC and its guide-strand binds to target mRNA to start mRNA cleavage.

mRNA site (usually in the coding region) determines how the shRNA regulates gene expression post-transcriptionally, i.e., primarily, mRNA cleavage or, manifested as off-target effects, mRNA degradation or inhibition of translation. The properties of the promoter are critical for an efficient shRNA expression system. Initially, polymerase III promoters, including U6 (7,8) and H1 (9), were used to drive shRNA expression. Since polymerase III promoters lack control of spatial and temporal gene expression and can compete for and occupy endogenous nuclear export mechanisms, polymerase II promoters have been used to construct gene expression systems for both *in vitro* as well as *in vivo* applications (10,11). In practice, several aspects of shRNA differ intrinsically from siRNA (12). First, less than 1% of duplex siRNA remains in the cells 48 h after administration due to the high rate of degradation and turnover. By contrast, shRNA is constantly synthesized in host cells, leading to more durable gene silencing. Since chemical modification is typically employed to improve stability and efficacy of siRNA, the bulk manufacturing of clinical-grade siRNA is cost-limiting compared with that of a shRNA expression vector. However, vector-based shRNA can only be modified by manipulating the expression strategy. In addition, the initiation of gene silencing may be affected by delayed nuclear processing of shRNA.

miRNA

MicroRNA (miRNA) is a class of highly conserved small non-coding RNAs which play an indispensable role in regulating cellular functions both physiologically and pathologically. miRNA is initially transcribed in the nucleus as a primary transcript (pri-miRNA), from precursors generally located within either intergenic regions or introns of protein coding genes and each pri-mRNA transcript may contain multiple pre-miRNA stem-loops (13). After being processed by Drosha, an RNase III endonuclease, pre-miRNA is exported to the cytoplasm and further cleaved by another RNase III enzyme Dicer to produce a 20–23 base-pair long mature miRNA comprised of guide and passenger strands with mismatches at the putative Ago2 passenger strand cleavage site. Processed mature miRNA is loaded into RISC to elicit translational inhibition with target mRNA degradation or sequestration in cytoplasmic P-bodies (14). Unlike siRNA and shRNA, which require a perfect match with the target mRNA, miRNA typically exerts translational inhibition by binding to partially complementary mRNA (most effectively to multiple miRNA binding sites in the 3′-UTR). As a result, the change in the expression of a single miRNA may affect hundreds of different genes since a perfect match is not a prerequisite for miRNA to function effectively (15,16).

miRNA modulates gene function in various ways. Originally, it was believed that miRNA binds to the 3′ UTR region of the target mRNA and executes its role in gene repression (13). Recently, it was found that miRNA can also identify the coding region (17), the 5′UTR region (18), or a combination of sites to inhibit gene expression although with less potency than at the 3′UTR . In addition to gene silencing, miRNA can promote protein translation by binding to the 5′UTR of the mRNA (19). miRNA also has the ability to bind to DNA and regulate gene expression. For instance, miR-373 was shown to activate gene expression by binding to the complementary promoter sequences of both E-cadherin and cold-shock domain-containing protein C2 (CSDC2) (20). These observations expand miRNA's working region and diversify its regulatory roles.

Bi-functional (bi) shRNA

A novel technology called bi-functional shRNA has recently been described (12). Bi-shRNA was developed to exploit both the cleavage and translational inhibition mechanisms of RNAi (12). It consists of two stem-loop shRNAs structures: one cleavage-dependent unit with perfectly matched passenger-strand and guide-strand, and one cleavage-independent unit composed of mismatched double strand. The two shRNA units are embedded in a miR-30 scaffold and are encoded by a plasmid vector. The mature transcript of the cleavage-dependent unit is loaded onto RISC incorporating Ago2, whereas the processed transcript of the cleavage-independent unit functions as an miRNA by binding to RISC sans cleavage of the guide strand due to the strategic placement of the mismatch site, incorporating Ago1,2,3,4 without endonuclease function, inducing mRNA degradation/p-body sequestration or translational inhibition. In principle, bi-functional shRNA is able to induce RNase-H like cleavage and non-cleavage mediated degradation of the target mRNA (decapping and deadenylation) and inhibit translation concurrently, leading to more rapid onset of gene silencing, higher efficacy and greater durability when compared with either siRNA or miRNA. The bi-functional approach has been applied to develop RNAi therapeutics for solid tumors with supporting data collected from cultured cells and xenograft tumor models (10,21).

THE APPLICATION OF siRNA AND shRNA IN CANCER

Due to their robustness and specificity, siRNA and shRNA have been extensively used to silence cancer-related targets. A large number of preclinical studies have presented favorable outcomes by silencing genes critical for tumor cell growth, metastasis, angiogenesis and chemoresistance.

For instance, metastatic pancreatic cancer is one of the most deadly cancers with the majority of patients presenting with unresectable disease and a 4–6 month median survival. Pancreatic duodenal homebox-1(PDX-1) has been found to be overexpressed in pancreatic adenocarcinoma and has the characteristics of an oncogene (22). PDX-1 belongs to a homeodomain-containing transcription factor family and plays primary roles in pancreatic organogenesis. In the adult pancreas, PDX-1 maintains beta-cell function by regulating transcription of insulin, glucokinase and glucose transporter type 2 (GLUT2). Silencing of PDX-1 expression represents an attractive approach to inhibit tumor growth in malignant pancreatic cancer. A plasmid vector encoding shRNA was used to knockdown PDX-1 expression in a pancreatic animal model. The shRNA plasmid was formulated with a DOTAP:cholesterol liposome delivery system and systemically administered to intraperitoneal PANC1 bearing SCID mice by tail vein injections every 2 weeks for a total of 3 injections. One month after the last injection, 4 of 5 mice receiving shRNA treatment showed no visible tumors, while mice receiving control vehicle treatment displayed multiple large tumors. Immunohistochemistry examination showed that the expression of PDX-1 was significantly reduced compared with the control group (23).

RNAi technology has been used to inhibit tumor metastasis. For example, in advanced prostate cancer, tumor cells frequently metastasize to bones and regional lymph nodes. A systemic delivery system has been developed to introduce siRNA to the bone-metastatic cancer sites using atelocollagen. siRNAs targeting either EZH2 or p110-alpha were formulated with atelocollagen and delivered to bone-metastatic lesions in a xenograft model by tail vein injection (24). The development of bone metastasis was monitored by bioluminescent imaging as the tumor xenograft was labeled with a luciferase reporter. Twenty-eight days after the treatment, animals treated with siRNA/atelocollagen complexes presented no increase of luminescence, whereas the control group had significant tumor metastasis in the thorax, jaws and legs. Furthermore, innate immune responses, monitored by serum IL-12 and IFN-alpha levels, were not observed in the siRNA-treated animals (24). Pin1 is a peptidyl-prolyl isomerase which catalyzes the cis/trans isomerization of peptidyl-prolyl peptide bonds. It is highly overexpressed in prostate and breast cancers. Pin1 may facilitate tumor growth and transformation by activating oncogenic signal pathways, including Ras, NF-kappa B and Rb. Retrovirus-encoded shRNA was used to silence Pin1 expression in a prostate cancer model. Pin1 shRNA significantly inhibited tumor growth, tumor metastasis and angiogenesis (25).

Angiogenesis is a critical factor for neoplasia and tumor metastasis. The VEGF pathway (VEGF and its receptors VEGFR1, 2, 3) is the most investigated pathway in angiogenesis. siRNA has been used to selectively silence VEGF and VEGF receptors to arrest tumor growth and angiogenesis. For example, the siRNA against human VEGF was intratumorally delivered to a prostate tumor xenograft model using an atelocollagen delivery system. The treatment regimen was every 10 days for a total of 4 times. Tumor growth was markedly suppressed in parallel with decreased microvessel density and VEGF expression (26). Moreover, the siRNA targeting VEGF receptor 2 (VEGFR2) was formulated with polyethyleneimine (PEI) nanoparticles, which were conjugated with peptide ligands to target integrin overexpressed on tumor neovasculatures. The nanoplexes were delivered to tumor xenografts via tail vein injection every 3 days. After two treatments, a significant inhibition of tumor growth and neovascularization was observed, accompanied with reduced VEGFR2 expression (27).

RNAi can be used to inhibit tumor growth in combination with chemotherapy or radiation therapy. For example, nuclear factor erythroid-2-related factor 2 (Nrf2) is a transcription factor which plays critical roles in maintaining redox homeostasis. An Nrf2 inhibitor, Kelch-like ECH-associated protein 1(Keap1), undergoes loss of function mutations in non-small cell lung cancers. As a result of Keap1 mutation, Nrf2 escapes from proteasomal degradation, accumulates in the nucleus, and induces a wide array of genes. The up-regulated genes modulate glutathione, thioredoxin and the drug efflux pathways thereby contributing to chemoresistance. Hence, Nrf2 was targeted by siRNA to increase sensitivity to chemotherapy by negatively regulating several drug detoxification enzymes and efflux proteins. Non-formulated siRNA was injected to the tumors in a lung cancer xenograft model, while carboplatin was respectively administered to the animals by intraperitoneal injection. The treatments were conducted twice a week for 4 weeks. It was reported that the siRNA targeting Nrf2 significantly inhibited tumor growth, while the combinational therapy of siRNA and carboplatin displayed a greater extent of tumor arrest compared with siRNA alone treatment. Importantly, the expression of Nrf2 and its downstream genes was significantly reduced compared with the control group (28).

THE APPLICATION OF bi-shRNA IN CANCER

The biochemical structure and gene-silencing characteristics of miRNA have been exploited to develop a novel shRNA expression system. A proprietary bi-functional shRNA expression platform has been developed to target driver genes and interacting dominant pathway and co-pathway nodes to inhibit tumor growth. Stathmin1 (STMN1) belongs to a family of microtubule-destabilizing proteins and plays a

critical role in the regulation of mitosis (29). STMN1 is highly expressed in solid tumors and its over-expression is correlated with poor prognosis in cancer therapy. We previously demonstrated marked antitumor effect in correlation with bi-functional RNAi induced knockdown of STMN1 *in vitro* in multiple tumor cell lines (10). We then tested anti-tumor activity of pbi-shSTMN1 in different xenograft models, including human colorectal tumor, primary human osteosarcoma, and primary human melanoma. The plasmid encoding bi-functional shRNA targeting human STMN1 was delivered as a DOTAP:cholesterol liposomal complex via intratumoral injection. In a human colorectal tumor xenograft model, 3 daily intratumoral injections of pbi-shSTMN1 reduced tumor growth by 57% 15 days after the last dosage accompanied by significant reduction of STMN1 protein. In addition, the intratumoral delivery of pbi-shSTMN1 induced 70% tumor volume reduction in a primary melanoma model and abrogated osteosarcoma xenograft growth. Moreover, safety assessment in STMN1 sequence-biorelevant rats demonstrated a maximum tolerated dose (MTD) of 92 μg (murine equivalent of 26.5 μg) following IV infusion. Substantial STMN1 expression knockdown in tumor cells was demonstrated at this dose and below, thus justifying clinical assessment. pbi-shSTMN1 in combination with docetaxel has been shown to enhance anti-tumor effect. Biodistribution studies have recently been completed, and a clinical IND for phase I trial is in preparation for submission to FDA.

miRNA AND CANCER

miRNA normally regulates gene expression controlling development, metabolism, immune response and aging (30–33). Deregulation of miRNA has been implicated in a wide array of human diseases, including cancer, neurological disease, cardiovascular disease, and diabetes (34). In cancers thus far studied, those miRNAs correlated with the malignant process can be either overexpressed or downregulated. A miRNA subtype normally functioning as a tumor suppressor can show reduced expression in tumors. For instance, miR-26a is highly expressed in normal tissues including the liver, but is downregulated in liver tumors. Patients with low miR-26a expression have decreased overall survival compared with those patients with high miR-26a expression (35). In addition, miR-34c, miR-145, and miR-142-5p also demonstrate tumor suppression properties in various lung cancers. Replacement of the downregulated miRNA significantly arrested the growth of lung cancer cells (36).

For miRNA with tumor-suppressing characteristics, a miRNA replacement therapy can be used to restore miRNA gene expression and inhibit tumor development. This approach is principally analogous to that used for siRNA/shRNA therapeutics except that miRNA mimetics are used to rescue miRNA expression. miRNA mimetics are similar to chemically modified siRNA and are introduced to target cells using identical delivery methods for siRNA therapeutics. For example, miR-34a is a tumor suppressor and is often lost in human cancers, notably, lung cancer and prostate cancer. Systemic delivery of synthetic miR-43a using a neutral lipid emulsion (NLE) led to the preferential accumulation of miR-34a in both normal lung tissues and orthotopic lung tumors, and the reduction of tumor volume in an autochthonous $KRAS^{G12D}$ mouse model of non-small cell lung cancer (NSCLC) (37). Moreover, miR-34a was identified as an inhibitor of prostate cancer progenitor cells and blocked metastasis of prostate cancer by directly repressing CD44 (38). MiR-16 is another tumor suppressor identified in prostate cancer. miR-16 regulates cell cycle progression and cell proliferation by targeting key cell-cycle genes such as CDK1 and CDK2. The systemic delivery of synthetic miR-16 using atelocollagen inhibited the growth of bone-metastatic prostate tumor in a mouse xenograft model (39). Mir-22 is able to repress tumor progression by inducing cellular senescence instead of apoptosis. MiR-22 regulates the senescence mechanism by targeting CDK6, SIRT1 and Sp1. Intratumoral delivery of synthetic miR-22 induced cellular senescence and inhibited tumor growth in a breast cancer xenograft model (40). In miRNA replacement therapy, plasmid or virus vectors are also utilized to deliver miRNA mimetics. For example, re-expression of miR-26a inhibited cyclin D2 and E2 and induced G1 arrest in liver cancer cells. miR-26a was then systemically delivered into a hepatocelluar tumor model using an adeno-associated virus and induced tumor-specific apoptosis and significant inhibition of tumor development without any observed toxicity (41).

In addition to its role as a tumor suppressor, miRNA can function as an oncogene (oncomiR). For example, miR-31 was identified as an oncogenic miRNA in lung cancers (42). It was reported that miR-31 and its target genes, including large tumor suppressor 2 (LATS2) and PP2A regulatory subunit B alpha isoform (PPP2R2A), were inversely expressed in mouse and human lung cancers. Silencing miR-31 expression repressed the growth of lung tumor cells, which could be reversed by co-expressing a pre-miR-31 miRNA or simultaneously inhibiting LATS2 and PPP2R2A expression. Likewise, mirR-155 was reported to be oncomiR in breast cancers (43). miR-155 was found to be overexpressed in breast cancers and exerted its oncogenic role by negatively regulating tumor-suppressor SOCS1, leading to constitutive activation of STAT3. Inflammatory stimulants, including IFN-gamma,IL-6 and LPS, upregulated the expression of miR-155, suggesting a linkage between inflammation and breast cancer.

In order to constitutively inhibit expression of oncogenic miRNA, antagomiRs are used to perfectly pair with oncogenic miRNAs and sequester them from binding to targets (44–46). Another strategy is to express transgene with multiple binding sites for an miRNA of interest to sponge endogenous miRNA expression (47,48), while miR-masking technology takes advantage of the high affinity between miRNA and the synthetic target-specific RNA oligonucleotide (49,50). Furthermore, small molecule inhibitors are used to selectively repress miRNA expression. For example, azobenzene is able to inhibit miR-21 expression (51).

RNAi AND CANCER IMMUNOTHERAPY

Immunotherapy has been extensively investigated in oncology in order to reactivate the restrained host immune system in cancer patients whether due to a focal or more extensive functional defect or an immune-edited malignant growth process. However, the progress in the development of cancer immunotherapy is limited due to the complex nature of immunosuppression and immunotolerance. RNA interference is being introduced to enhance antitumor immunity for more efficient and personalized cancer therapeutics.

RNAi has been employed to block immunosuppressive pathways in dendritic cells, T cells and tumor cells. For instance, siRNA has been used to knockdown expression of 3 inducible proteasome subunits in mature dendritic cells (DCs). The transfected DCs had increased expression of constitutive proteasomes, leading to an altered repertoire of tumor antigenic peptides. The DCs generated from melanoma patients were transfected with the immunoproteasome siRNA and subsequently induced antigen-specific CTL activity against autologous melanoma cells (52). Suppressor of cytokine signaling 1(SOCS1) negatively regulates JAK/STAT pathway in T cells, DCs and other immune cells. It specifically attenuates the extent of antigen presentation in DCs. Silencing SOCS1 in DCs using lentivirus-encoded shRNA stimulated antigen presentation and elicited antigen-specific CTL activity. Moreover, in a mouse melanoma xenograft model, immunization with DCs previously transduced with SOCS1 shRNA induced potent antigen-specific anti-tumor immunity and inhibited tumor growth (53). Likewise, zinc-finger protein A20 is an ubiquitin-modifying enzyme and downregulates TNFR and TLR signaling pathways. Silencing A20 with shRNA delivered by lentivirus increased the expression of costimulator molecules and proinflammatory cytokines, and abolished regulatory T cells (Treg cells)-mediated suppression in an antigen-specific manner, Immunization with A20-silenced DCs elicited antitumor immunity, a result of tumor infiltration of suppressed Treg cells and hyperactivated CTLs and T helper cells (54).

RNAi has the potential to lessen the immunotolerance, which allows tumors to escape immune surveillance. IDO is an enzyme that catalyzes the degradation of tryptophan, an essential amino acid for T cell viability and proliferation. It suppresses immune response by inducing T cell apoptosis. IDO is constitutively expressed in most human tumors (55). Silencing of IDO reduced T cells apoptosis and enhanced T cell proliferation. Intratumoral delivery of IDO siRNA significantly reduced tumor growth in a melanoma xenograft model (56).

Besides the adaptive immune system, the innate immune response can be harnessed to increase tumor cell immunogenicity. For example, a Bcl-2-specific siRNA with 5′-triphosphate ends combined the activation of the innate immune response with target-specific gene silencing. Recognition of 5′-triphosphate by antiviral helicase retinoic acid-induced protein I (Rig-I) induced type I IFN and activated NK cells in tumors, synergized with siRNA-mediated Bcl-2 gene silencing. This combinatorial approach was validated in various animal models and human melanoma cells (57). In another study, a siRNA targeting Stat3 was synthetically linked to an oligonucleotide agonist of toll-like receptor 9 (TLR9) to simultaneously induce antitumor immunity and gene silencing in tumor–associated TLR9+ myeloid cells and B cells. Intratumor and systemic delivery of CpG-Stat3 siRNA reduced tumor growth in xenograft melanoma models (58).

CHALLENGES OF RNAi THERAPEUTICS DEVELOPMENT

Despite RNAi's immense potential in clinical applications, several hurdles have to be overcome for RNAi-based therapies to move from the bench to the clinic. First, an efficient delivery approach needs to be developed to bring RNAi effector molecules to the target cells. Second, RNAi induces an innate immune response, which is a particular concern when considering siRNA. In addition, sequence and non-sequence related off-target effects have to be carefully measured since RNAi has the potential to knockdown non-targeted genes and, by saturating endogenous miRNA nuclear export and Ago2 proteins, elicit unwonted cytotoxicity. Finally, the molecular mechanism and pharmacokinetics data have to be profiled for regulatory filings associated with clinical testing.

Delivery

Insofar as anionic siRNA or DNA vectors cannot pass through the cell membrane without the aid of delivery vehicles, nanoparticles can be used to formulate RNAi molecules that can be efficiently delivered into the target

cells. Nanoparticles, between 1 and 100 nm in size, enter cells via endocytosis. Once taken up into the endosome, the siRNA or plasmid has to escape from endosome to avoid subsequent degradation by lysosome. Thus, endosome release is a key factor in formulating efficient delivery platforms (59). Generally, non-viral delivery systems such as nanoparticles between 15nm and 100nm are optimal for systemic delivery (60), balancing pharmacokinetics and Enhanced Permeability Retention (EPR) effect. However, enhanced flexibility of delivery vehicles overrides the limitation imposed by size (or molecular weight) considerations. For example, DOTAP:cholesterol bilamellar invaginated vesicles (BIVs) encasing DNA in the range of 200-450nm produced the highest gene expression after intravenous injection in comparison with other vehicles. In addition, these larger but more flexible lipoplexes (due to the properties of DOTAP and cholesterol) are stable in >70% serum and have what appears to be an optimized half life of ~5 h (61).

Nanoparticles can be modified on the surface to improve their interactions with cancerous cells. Receptors and epitopes, highly expressed in cancer cells but not in normal cells, can be exploited to develop targeted nanoparticle delivery systems. This would allow the formulated RNAi molecules to be taken up selectively by the tumor cells minimizing side effects on the normal cells. For example, cyclodextrins are cyclic oligomers of glucose and are complexed with transferrin in a nanoparticle delivery system to formulate synthetic siRNA. The same formulation has been successfully used in both primates and human patients (62). Chitosan is another candidate compound with low immunogenicity and high delivery efficacy. Arg-Gly-Asp (RGD) peptide-labeled chitosan nanoparticles (RGD-CH-NP) selectively delivered siRNA into tumor–associated endothelial cells (63).

Small molecule bipeptide β turn mimetics have also been used for the targeted delivery of RNAi cancer therapeutics (64). A small molecule library, based on secondary structure motifs of protein-ligand interactions, was screened *in vitro* for compounds which were able to selectively bind to tumor-associated endothelial cells, but not normal endothelial cells or cancerous cells. The identified candidates were complexed with bilamellar invaginated vesicle liposomes and intravenously administered to a xenograft tumor model. This liposome targeted delivery system increased efficiency of delivery to the tumor microenvironment by 200 fold (64). The same technology has been applied to selectively deliver plasmids encoding bi-functional shRNA to human pancreatic cancers and human melanoma cancers (21).

Innate Immune Response and Off-Target Effects

The potential for RNAi immune mediated and sequence-related and—independent off-target side effects requires that the safety profile of this class of agents be thoroughly evaluated before moving into the clinical arena. The innate immune response is a well described mechanism of RNAi-related toxicity. The innate immune response can be evaluated by measuring a panel of inflammatory markers, including IFN alpha and IFN beta, cytokines (such as IL-6 and TNF alpha), and chemokines induced by TLR agonists.

Synthetic siRNA duplexes are potent activators of the innate immune response in mammals (65,66). The extent of innate immunity induction is determined by the siRNA structure, siRNA sequence, delivery vehicle, delivery route, cell type and species. The innate immune response elicited by siRNA is mainly mediated through Toll-like receptor (TLR) pathways. Three TLRs, including TLR7, TLR 8 and TLR3, have been reported to be activated by the siRNA duplex. TLR7 and TLR 8 are localized in the endosome and their activation is highly dependent on siRNA sequence and ribonucleotide structure (67). Since TLR7/8 signaling represent the majority of innate immune response induced by siRNA in animals, the siRNA target selection is particularly critical to avoid sequences that have the potential to activate TLR7 and TLR8. Chemical modifications, such as 2′-O-ribose methylation, have been introduced to minimize if not inhibit the innate immune response *in vivo*. In addition, early endosomal escape can also help decrease the innate immune response as it reduces the extent of engagement between siRNA and endosomal TLR receptors. Unlike TLR7/8, TLR3 is expressed both in endosome and on cell surface.Its activation seems to be less dependent on siRNA sequence. For instance, it was reported that siRNA might bind to cell surface TLR3 receptors and elicit innate immune responses independent of RNAi mechanism. Activated TLR3 receptors induced antiangiogenic effects through activation of IFN-gammaand IL-12, independent of the siRNA sequence and proposed targets (68). Also of note is that siRNA duplex can elicit innate immune responses through non-TLR-mediated pathways, such as dsRNA-activated protein kinase (PKR) and retinoid-inducible gene 1 (RIG1). PKR is believed to be only activated by viral dsRNAs or other dsRNAs, such as double-stranded RNAi effectors. Upon activation, PKR phosphorylated eIF2 alpha and I kappa B, leading to global inhibition of translation and NF-kappa B-mediated upregulation of interferon cytokines. RIG1 is a cytoplasmic RNA helicase that is able to sense either ssRNA or dsRNA containing uncapped 5′-triphosphate. Activated RIG1 relays signals to downstream targets, including IRF3/5/7 and NF-kappa B, inducing the production of interferons and other inflammatory cytokines. The recognition of RNAi effectors by PKR or RIG1 does not depend on RNA sequence, but is potentially determined by RNA structure (67).Since vector-encoded shRNA and bi-shRNA

are initially transcribed in the nucleus and then transported to the cytoplasm, the presence of endosomal TLR 3, 7 and 8 activation is moot. However, they may induce the innate immune response by 1) endosomal TLR 9 CpG methylation dependent activation by plasmid encoding shRNA, (62) 2) via dsDNA cytoplasmic sensors (DAI, DNA-dependent activator of IFN-regulatory factors) and AIM2 (absent in melanoma), and 3) following processing and nuclear export, via PKR and RIG1pathways as discussed above. shRNA and bi-shRNA seem to be less potent activators of innate immunity than siRNA based on their mechanisms and numerous preclinical studies.

The specificity of RNAi is based on sequence homology between small RNA and target mRNA. However, the partial homology between small RNA and unintended mRNA transcripts might result in non-desirable gene silencing. In principle, the "seed region", bases 2-7[8] from 5′ end of the guide strand, appears to be the major determinant of potential off-target silencing. Hence, the cross-homology possibility of the seed region (including the number of target sequences as well as their site), has to be thoroughly considered during the RNAi drug design for clinical applications (69,70).

Saturation of miRNA Machinery

Perturbation of endogenous miRNA machinery may also contribute to shRNA-related toxicities. Systemic delivery of shRNA encoded by adeno-associated virus (AAV) resulted in profound toxicities in mice, notably hepatotoxicity, a result of endogenous miRNA pathway oversaturation (71). Furthermore, neuronal toxicities in striatum were found in mice receiving locally delivered AAV vector encoding U6-shRNA (72). The neurotoxicity was attributed to robust shRNA expression and when an artificial miRNA backbone was used to encode the same shRNA expression cassette, the toxicities were significantly attenuated without compromising RNAi efficacy. It was speculated that the artificial miRNA cassette was expressed at a lower level but was processed more efficiently, which led to less saturation and toxicity. Saturation-induced toxicities appear to be exclusive to the DNA-encoded shRNA. Systemic delivery of synthetic siRNA did not disturb cellular miRNA biogenesis or function and cause cellular toxicities (73). One possible explanation is that the siRNA silencing mechanism is mediated downstream of the miRNA biogenesis pathway, which is otherwise crucial for efficient processing of shRNA.

The saturation mechanism has been investigated further and involves several identified components. Exportin-5 (*XPO5*) is a transporter which shuttles pre-miRNA or exogenous shRNA from nucleus to cytoplasm (74). RNAi pathway products produced by shRNA regulated by a pol II promoter significantly saturated XPO5 and Ago2 which was prevented by the use of either weak pol III promoters (74) or pol II promoters (75) which also use the CRM1 export pathway. Overexpression of Ago2 also attenuated cytotoxicity and improved persistence of shRNA (62).

Monitoring Assays of RNAi Activity

Quantitative measurement of RNAi effector molecules in pharmacokinetics study is critical to the successful development of RNAi-based therapeutics. With more RNAi-based therapies moving from the bench to the clinic, a reliable quantification method is needed to examine the intracellular concentration of siRNA/shRNA in order to evaluate the delivery efficiency and the pharmacokinetics of RNAi drugs. Several bioanalytical approaches are being utilized to determine RNAi effector molecules in preclinical and clinical studies, including quantitative RT-PCR, hybridization assay, HPLC and LC-MS (76). Among them, quantitative PCR has been extensively utilized to investigate pharmacokinetic profiles of RNAi therapeutics in preclinical and clinical development (77–80). Taking advantage of a hairpin-containing RT primer, this technique is able to detect nucleotide sequences as short as 19 bases (81), hence qualifying for quantitatively measurement of small RNAs, including siRNA, shRNA and miRNA. Stem-loop qRT-PCR has superseded conventional hybridization-based techniques due to increased sensitivity, specificity as well as wider dynamic range and ease of use. Therefore, stem-loop qRT-PCR has been developed to quantitatively measure small RNAs to evaluate the biodistribution and kinetics of RNAi pharmaceuticals. In a study aimed to treat solid tumors involving liver, as early as 0.5 h after systemic delivery using lipid nanoparticles (LNP), chemically stabilized siRNA was detected in liver, spleen and kidney (ranked as high level to low level), with negligible levels in lung, heart, duodenum and brain. The siRNA levels in liver and spleen decreased by 10–fold 24 h after the dosage (52). Furthermore, great efforts have been directed to deliver RNAi molecules to other organs. Synthetic mir-124 mimics was complexed with a neutral lipid emulsion (NLE) and then intravenously administered to mice via vein-tail injection. Ten minutes after injection, miR-124 mimics was detected in all 4 tissues tested, including blood, lung, kidney and liver, with a preferential accumulation in the lung over the liver (37).

Investigation of molecular mechanism of gene silencing is also necessary in development of RNAi therapeutics. 5′ RACE-PCR assay has been adopted as a standard assay in RNAi therapeutic development. This assay is a modified version of *r*apid *a*mplification of *c*DNA *e*nds (RACE) and was first used to identify cleavage products of siRNA targeting apolipoprotein B (apoB) after intravenous injection into mice (82). Since then, it has been widely used in

cultured cells, animal models and clinical trials (10,62,83). However, RACE-PCR is not able to quantify the cleavage product of gene silencing due to its inherent limitation. RNA sequencing may be used in the future since it can examine the identity and abundance of transcripts simultaneously.

RNAi-BASED CANCER THERAPEUTICS IN CLINICAL TRIALS

Despite the numerous RNAi therapeutics reported in preclinical development, only a few RNAi-based drugs for cancer have translated into the clinic (Table 1). Thus far, all appear to be well tolerated and no dose-limiting toxicities have been reported.

Calando Pharmaceuticals reported their study results in human patients with solid tumors in March of 2010 (62), which is believed to be the first proof-of-concept study for efficacy in target gene knockdown with systemically administered siRNA in humans. In this study, an unmodified siRNA targeting ribonucleotide reductase M2 (RRM2) was formulated with cyclodextrin-containing polymer nanoparticles, and then was administered intravenously to patients with metastatic melanoma. siRNA-loaded nanoparticles accumulated in tumor cells in a dose-dependent manner. A statistically significant reduction of both RRM2 mRNA and protein was found when compared with predosing tumor tissues. The predicted cleavage product of RRM2 mRNA was detected from one patient who received the highest dose of siRNA nanoparticles (30 mg m^{-2}). Furthermore, the safety profiles showed that the administered siRNA was well tolerated without dose-limiting toxicity. Despite the promising data, the effects on tumor reduction or clinical phenotypes were not available at the time of publication.

As mentioned above, siRNA requires chemical modification to increase serum stability, cellular uptake and duration of action. Alnylam has employed chemically modified siRNA to treat a variety of diseases, including liver cancer. Chemically modified siRNA was formulated with a proprietary LNP lipoplex which delivered more than 90% of lipoplex to the liver according to Alnylam's study reports. Two modified siRNAs targeting either the kinesin spindle protein or vascular endothelial growth factor were formulated together with proprietary LNP technology and systemically administered by IV infusion. This RNAi therapy was positioned to treat advanced solid tumors with liver involvement due to high delivery efficiency of LNP to the liver. However, for effective therapy of metastatic foci, it will be critically important to deliver RNAi effector molecules to other organs in addition to the liver. Thirty-one patients with multiple prior therapies, a majority of them with colorectal cancer, were administered ALN-

Table I Clinical Trials of RNAi-Based Cancer Therapeutics

Drug	CALAA-01	ALN-VSP02	TKM-080301	n/a	Atu027	EZN-2968	FANG™ vaccine
Phase	I	I	I	n/a	I	I	I
Tumor Type	solid tumor	advanced solid tumor with liver involvement	solid tumor or lymphoma	astrocytic tumor	advanced solid tumor	advanced solid tumor or lymphoma	advanced solid tumor
Delivery	cyclodextrin-containing polymer nanoparticle	lipid nanoparticle	lipid nanoparticle	naked	lipoplex	naked	ex-vivo by electroporation
Route	IV infusion	IV infusion	IV infusion	local, brain	IV infusion	IV infusion	intradermal
Target	ribonucleotide reductase M2 (RRM2)	kinesin spindle protein and VEGF	polo-like kinase 1 (PLK1)	tenascin-C	protein kinase N3	HIF-1 alpha	furin
RNAi	unmodified siRNA	chemically modified siRNA	chemically modified siRNA	160bp double-stranded RNA	chemically modified siRNA	LNA antisense oligonucleotide	bi-shRNA
Current Enrollment	(36)	(41)	(42)	(53)	(33)	(59)	(59)
Company	Calando	Alnylam	Tekmira	Polish Academy of Sciences (Poland)	Silence Therapeutics AG (Germany)	Enzon	Gradalis
References	(62), a	(77), a	a	(90)	(84), a	(85), a	(86), a

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VSP02 with doses ranging from 0.1 to 1.5 mg/kg. This treatment was generally well-tolerated and.no dose-dependent changes in liver function test were reported 46.6% of patients (7/15) had stable disease (SD) for at least 2 months at dose ≥0.7 mg/kg, compared with 8.3% (1 of 12) with SD at doses ≤0.4 mg/kg. Cleavage of VEGF mRNA was identified in two liver tumor biopsies and one extrahepatic tumor. Pharmacologically relevant concentrations (0.3-142 ng/g tissue) of both VEGF and KSP siRNAs were detected in tumor biopsies (77).

Chemically modified 23 base-pair blunt-end siRNA has been utilized by Silence Therapeutics to treat solid tumors, including metastatic tumors in the lung and the breast. The proprietary AtuPlex technology is employed to deliver siRNA, which resides on the surface of the positively charged and pegylated liposomes, preferentially o the endothelium. Atu027, a product currently in a phase I trial, was developed to knockdown the expression of protein kinase N3 (PKN3), which is involved in the progression and metastasis of solid tumors (84).

In addition to solid tumors, the principle of gene silencing has been exploited to treat lymphoma by Enzon Pharmaceuticals. Locked nucleic acid antisense oligonucleotides targeting HIF-1 alpha are being evaluated in phase I studies in patients with lymphoma or advanced solid tumor. Preliminary results showed stable disease in two patients and no dose-limiting toxicities were observed (85).

Besides being evaluated as a systemic targeted therapeutic, RNA interference has been used to develop vaccines in cancer immunotherapy. For example, an autologous whole cell tumor vaccine incorporating a bi-functional shRNA has recently completed Phase I evaluation (86). Briefly, freshly harvested tumor cells from patients were disaggregated and electroporated with an expression vector encoding both rhGM-CSF and bi-functional shRNA targeting furin (FANG™). The FANG™ treated tumor cells were irradiated and then intradermally administered to the patients. Furin, a member of the subtilisin-like proprotein convertase family, is overexpressed in solid tumors and is the dominant proprotein convertase for the activation of the endogenous immunosuppressive transforming growth factor beta isoforms, TGFβ1 and TGFβ2. The reduced levels of furin protein also impact (by feedback regulation) the expression of TGFβ1 and TGFβ2 mRNA, the conversion of the proform of TGFβ1 and TGFβ2 protein into the mature (active) form of their respective proteins and, by interfering with the TGFβ-furin amplification loop, further dampen the expression of furin itself (87–89). The overall reduced expression of TGFβ1 and TGFβ2 both inhibit local immunosuppression and promote tumor surface antigen and MHC protein displays (unpublished results). The FANG™ vaccine has just completed Phase I clinical trial and the study report is expected to be presented in 2011.

CONCLUSION

Over the past decade, our understanding of RNAi has significantly increased and, with new insight into construction and mechanisms of action and toxicity, the application of RNAi in has evolved from the bench to the cancer clinic. The cumulative preliminary results from several clinical trials continue to confirm the safety of both locally and systemically administered RNAi-based cancer therapeutics. The demonstration of RNAi efficacy in cancer patients is expected emerge in the coming years. Despite these achievements, the effective and efficient clinical application of RNA-based therapeutics will need to overcome remaining limitations, including target selection, delivery design, off-target effects and, in certain instances, activation of the innate immune response. Novel RNAi and delivery vehicle design, as exemplified by bi-functional shRNA and the DOTAP: cholesterol BIV , will hopefully expedite the development of effective clinically applicable cancer therapeutics.

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